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Immuno-chemical Studies on the Alkali-labile Carbohydrate Chains of Human Serum Glycoproteins

By *G. Uhlenbruck, I. Reese, P. Vaith* and *H. Haupt*

Medical University-Clinic Cologne and Behringwerke AG, Marburg, Germany

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Summary: Human serum glycoproteins can be classified into those containing N-acetyl-*D*-galactosamine and into those lacking this hexosamine. The N-acetyl-*D*-galactosamine-containing serum glycoproteins have alkali-labile chains containing this hexosamine linked O-glycosidically to hydroxy amino acids. These alkali-labile chains can be demonstrated in neuraminic acid free serum glycoproteins by gas liquid chromatography and by using precipitating lectins from invertebrates and plants. They are represented by two chains, one containing only N-acetyl-*D*-galactosamine, the other with *D*-galactose linked (1–3) β-glycosidically to this hexosamine forming a disaccharide. Serologically these two chains, which usually occur together on one molecule, can be characterized by their reaction with lectins from *Helix pomatia* (anti-A like) and from *Agaricus bisporus* and *Arachis hypogaea* (anti-TF specificity).

Immunchemische Untersuchungen an alkali-labilen Kohlenhydratketten menschlicher Serum-Glykoproteine

Zusammenfassung: Menschliche Serum-Glykoproteine kann man in zwei Gruppen einteilen, nämlich solche, welche N-Acetyl-*D*-Galactosamin enthalten, und in solche, die dieses Hexosamin nicht besitzen. Die N-Acetyl-*D*-Galactosamin enthaltenden Serum-Glykoproteine haben diesen Aminozucker O-glykosidisch an Hydroxy-Aminosäuren gebunden. Man kann diese alkali-labilen Ketten in neuraminsäure-freien Glykoproteinen des Serums nachweisen und zwar mit Hilfe der Gaschromatographie und durch präzipitierende Lektine aus Invertebraten und Pflanzen. Sie bestehen aus zwei Ketten-Typen, eine davon enthält nur N-Acetyl-*D*-Galactosamin, während die andere noch *D*-Galactose (1–3) β-glykosidisch an dieses Hexosamin gebunden besitzt, so daß ein Disaccharid entsteht. Serologisch können diese beiden Ketten, die in der Regel gemeinsam auf einem Molekül vorkommen, durch ihre Reaktionen mit den Lektinen aus *Helix pomatia* (Anti-A ähnlich) und denen aus *Agaricus bisporus* und *Arachis hypogaea* (Anti-TF Spezifität) charakterisiert werden.

Introduction

In foregoing papers of this series concerning the chemical structure and the serological role of human asialo serum glycoprotein oligo-saccharide chains, we have already investigated and described:

1. The reaction of neuraminic acid-free serum glycoproteins with various, mainly anti-β-*D*-galactosyl specific lectins (1).
2. The fact that some of these lectin receptors are identical with two alkali-labile bound carbohydrate chains: Chain I consisting of the disaccharide β-*D*-galactosyl-(1–3)-N-acetyl-*D*-galactosamine, and chain II represented by α-linked N-acetyl-*D*-galactosamine. Both chains are bound O-glycosidically to serine or threonine of the protein backbone (2).
3. These two chains could be clearly identified by their specific reaction with special purified lectins: Chain I reacts with the lectins from *Arachis hypogaea* (peanut receptor or TF-antigen) and *Agaricus bisporus*, whereas chain II reacts with the one from *Helix pomatia* (the so-called 'A-like' receptor) (2).

In continuing this work, which has been undertaken and planned in order to investigate the contribution of the different carbohydrate chains to the "liver clearance" and metabolism of asialo glycoproteins as suggested by *Ashwell* (3, 4), we have now focussed our research on the following problems, the results of which we will discuss here:

a) The distribution of alkali-stable and alkali-labile carbohydrate chains within the group of serum glycoproteins.

b) The chemical characterisation of the alkali-labile chains I and II by gas-liquid chromatography and

c) the serological confirmation of these data by using the specific lectins, mentioned already under point 3.

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Material and Methods

Material

Human serum glycoproteins were highly purified by *Haupt* et al. and obtained from Behringwerke AG, Marburg (West Germany).

Lectins: Their preparation is described in a previous paper (2).

Techniques

Gas liquid chromatography (GLC)

a) Analysis of hexoses and hexosamines
Glycoprotein samples (2–3 mg) were hydrolysed in 3 mol/l HCl at 100 °C for 4 hours. After adding 30 µg erythritol as an internal standard, the solution was "neutralized" by $AgNO_3$, the sediment was removed by centrifugation and washed 3 times with distilled water. The combined supernatant and washings were lyophilized and then reacetylated by incubation with acetic anhydride for 16 h at room temperature (5). After relyophilization the sample has to stay in vacuo over $CaCl_2$ for 24 h. In order to prepare the samples for analysis in the gas-liquid chromatograph, they were trimethylsilylated for 30 min, as described by *Sweeley* et al. (6), except we used hexamethyldisilazane and trimethylchlorosilane in the reaction mixture.

The data of the gas-liquid chromatograph we used and the condition of the analysis have been described in a previous paper (7).

b) Analysis of the disaccharide
The glycoproteins were desialylated by mild acid hydrolysis (incubation with 0.05 mol/l H_2SO_4 for 1 h by 80 °C) followed by alkaline borohydride treatment as performed by *Newman* et al. (7). The disaccharide content of the glycoproteins was assayed by GLC in the same way as the monosaccharides, except that they were trimethylsilylated for 2 h and chromatographed isothermally at 250 °C with trehalose as an internal standard.

c) Estimation of alkali-labile bound N-acetyl-galactosamine
This procedure was performed analogous to the disaccharide analysis by mild acid hydrolysis and subsequent alkaline borohydride treatment, by which the N-acetyl-galactosamine is converted into galactosaminitol, which can be detected by GLC (temperature programme 125 °C – 230 °C, increasing 4 °C/min), internal standard: instead of trehalose erythritol.

Chemical analysis of sialic acid

The sialic acid (N-acetyl-neuraminic acid) was estimated by using *Bial*'s orcinol reagent (8). N-acetyl-neuraminic acid was taken as a standard.

Haemagglutination inhibition assay

The assay was performed by using the *Cooke* serial dilution microtitre system (Cooke Instruments, Zollikon, Switzerland).

Red cells were treated with neuraminidase for this purpose and desialylated serum glycoproteins were used. The latter was achieved by incubating 5 mg/ml protein in saline with 50 µl neuraminidase (500 units/ml, Behringwerke AG, Marburg) for 1 h at 37 °C. The test red cells were made up to a 2% suspension in saline (erythrocytes of group 0 were taken from a single, "standard" person) after they had been treated with neuraminidase (200 µl neuraminidase to 20 ml 2% red cell suspension) for 1 h at 37 °C and washed three times with saline.

The concentration of the lectins was prepared in such a way, that a dilution of an agglutinatory dose of 1:4 still gave a good clumping picture. The haemagglutination inhibition assay titer was defined as the minimum amount of substance inhibiting the agglutination of red cells by the lectin at an agglutinatory dosis of 4.

Results

The monosaccharide analysis of all investigated serum glycoproteins is listed in table 1. According to this table one may classify serum glycoproteins into two groups: one group containing N-acetyl-*D*-galactosamine, the other not. We could find no serological (lectins), or gas chromatographic evidence for the occurrence of alkali-labile carbohydrate chains in the last group. By definition, alkali-labile carbohydrate chains are linked via N-acetyl-*D*-galactosamine either to serine or to threonine O-glycosidically; thus this last group does not contain any alkali-labile carbohydrate chains and was excluded from further experiments.

Accordingly, as has been outlined in figure 1, we investigated only the first group of serum glycoproteins, namely those containing N-acetyl-*D*-galactosamine. As shown by the presence of N-acetyl-*D*-glucosamine and *D*-mannose, typical markers for alkali-stable carbohydrate chains, alkali-stable carbohydrate chains are also formed in these glycoproteins.

The presence of alkali-labile carbohydrate chains I and II was then verified by serological and chemical methods in this class of glycoproteins. The results of our experiments are documented in table 2 and 3. However there is no true relationship between the total content of N-acetyl-*D*-galactosamine and the quantity of the same hexosamine as quantitatively detected in the two alkali-labile chains. This mis-relation must be attributed to the alkaline borohydride treatment of the glycoproteins, which leads to a so-called "peeling-reaction" by more or less splitting N-acetyl-*D*-galactosamine molecules into pieces, which can no longer be identified by GLC (9, 10, 11).

The results of our investigations (as shown in tables 2 and 3) can be summarized as follows:

1. Of the 32 human serum glycoproteins we investigated only nine contain N-acetyl-*D*-galactosamine-containing alkali-labile carbohydrate chains. In the native state, these residues will be more or less or even completely, substituted or serologically blocked by N-acetyl-neuraminic acid (2). The other serum glycoproteins do not contain such structures.
2. With the exception of α_2HS-glycoprotein, all these asialo-glycoproteins react with lectins from peanut, *Arachis hypogaea*, in the precipitin reaction (2). In the haemagglutination inhibition assay, fetuin, β_2-glycoprotein III and IgD do not exhibit any inhibition, obviously due to the small amount of the TF-disaccharide chain I (see introduction), whereas in the agar gel diffusion much higher concentrations can be used and have been used (up to 5%). On the other hand, the competing TF-receptor on the erythrocyte surface has been shown to have a much higher affinity to the lectin than the few groups on the inhibitory substance. Serological unreactivity may also be due to steric or topochemical reasons...

Tab. 1. Carbohydrate content of some human serum glycoproteins in %.

	GalNac	GlcNac	Fuc	Man	Gal	Sialic Acid
Cholinesterase	4.3	8.8	0.2	3.1	3.5	6.0
α_2HS-Glycoprotein	1.2	2.8	0.1	2.8	2	4.8
C_1-Inactivator	5.2	9.7	0.4	4.6	6.1	13.1
8Sα_3-Glycoprotein	1.6	12.4	0.4	6	3.4	8.5
Inter-α-Trypsin Inhibitor	2.8	1.3	0.1	1.1	0.7	2
Fetuin*	1.1	6	–	1.7	3.7	7.8
β_2-Glycoprotein III	1.1	2.3	0.1	1.1	1.5	3.2
Ig A	0.9	2	0.15	1.3	1	1.8
Ig D	1	3	0.1	1.4	1.1	1.6
Antithrombin III	–	5.6	0.1	3.7	3.7	3
α_2-Macroglobulin	–	2.8	0.3	2.5	1.5	1.8
"Secretory component"**	–	9.8	3	4.1	4.5	2
Ig G	–	1.6	0.1	0.5	0.7	0.2
Ig M	–	3	0.9	1.9	1	1.2
Ig E	–	5	0.4	1.7	2.9	1.8
Hemopexin	–	4.5	0.2	3.9	2.8	7
Transferrin	–	1.8	–	1.7	0.7	1.4
α_1 acid-Glycoprotein	–	16.2	0.7	6.7	7.1	11
α_1B-Glycoprotein	–	3.9	0.1	1.6	2.5	4.1
3, 1Sα_2-Glycoprotein	–	6.1	0.4	3.2	3.4	5.6
α_1-Antitrypsin	–	5.2	0.1	4.5	3.0	3.9
Gc-Globulin	–	1.4	0.1	0.6	0.7	–
Prothrombin	–	2.4	–	1.4	1.4	4
C_3-Activator	–	5.1	0.1	2.2	1.4	1.6
3, 8Sα_2-Glycoprotein	–	6.5	0.1	4.3	2.1	3.5
9, 5Sα_1-Glycoprotein	–	4.7	0.1	0.9	1.4	4
Haptoglobin	–	8.1	0.7	3	3.1	5
Thyroxin Binding Globulin	–	6.5	0.2	3.3	2.9	3.7
β_2-Glycoprotein I	–	8.2	0.2	3.8	5.7	4.8
Coeruloplasmin	–	5.2	0.2	1.4	1.5	2.1
α_1-Antichymotrypsin	–	6.4	0.2	4.3	5.2	7
C_1q***	–	1.5	0.2	0.6	2.3	0.2

* Fetuin is taken from fetal calf serum
** Secretory component is taken from human colostrum
*** C_1q also contains 2.2% glucose

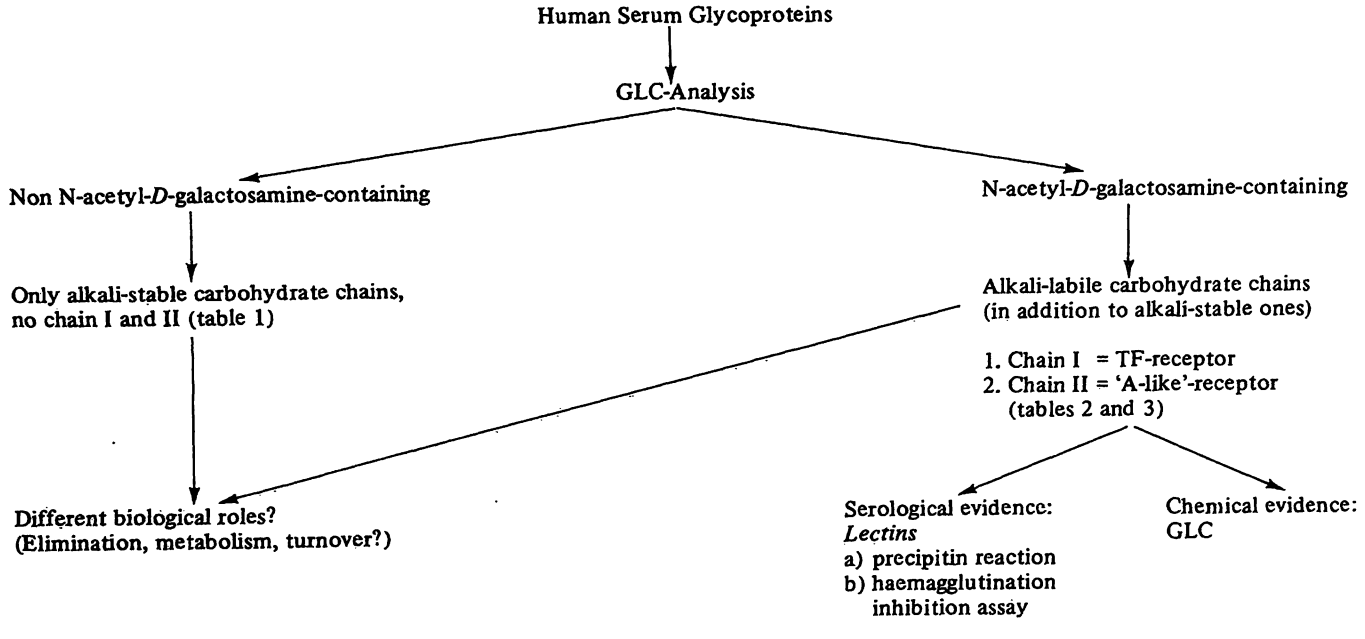

Fig. 1. Alkali-stable and alkali-labile carbohydrate chains of human serum glycoproteins.
As can be deduced from table 2 and 3, all serum glycoproteins, with the exception of α_2HS-glycoprotein and Ig D of the right group have both types of alkali-labile chains.

Tab. 2. Carbohydrate chains in different N-acetyl-*D*-galactosamine-containing desialylated human serum glycoproteins.

A. The TF-Receptor

Serum glycoprotein	I. Serology: a) Haemagglutination-Inhibition-Assay		b) Precipitin-Reaction in Agar-Gel (2)		II. Chemical Estimation: Gas-Liquid Chromatography		
	Arachis hypogaea	*Agaricus bisporus*	*Arachis hypogaea*	*Agaricus bisporus*	Disaccharide-Content (%)	Disaccharide-Content (mol/mol protein)	N-Ac-Gal-Content (mol/mol protein)
Cholinesterase	2^9	2^4	++	++	4.3	38.9	68.3
α_2HS-Glycoprotein	–	2^4	–	++	1.6	2	2.7
C_1-Inactivator	2^7	2^4	++	++	1.4	3.8	23.4
8Sα_3-Glycoprotein	2^6	2^4	++	++	0.6	3.45	16
Ig A	2^3	2^5	++	++	0.4	1.7	6.5
Inter-α-Trypsin Inhibitor	2^5	2^4	++	–	0.3	1.2	9
Fetuin*	–	2^4	++	–	0.2	0.3	2.5
β_2-Glycoprotein III	–	2^2	++	+	0.2	0.2	1.7
Ig D	–	–	++	++	0.1	0.44	7

* Fetuin is taken from fetal calf serum

Structure suggested for the receptor of the lectins from *Arachis hypogaea* and *Agaricus bisporus*: β-*D*-galactosyl(1-3)-N-acetyl-*D*-galactosamine → serine (threonine)

Tab. 3. Carbohydrate chains in different N-acetyl-*D*-galactosamine-containing desialylated human serum glycoproteins.

B. The 'A-like'-Receptor

Serum glycoprotein	I. Serology: a) Haemagglutination-Inhibition-Assay	b) Precipitin-Reaction in Agar-Gel (2)	II. Chemical Estimation: Gas-Liquid Chromatography	
	Helix pomatia	*Helix pomatia*	Galactosaminitol-Content (%)	Galactosaminitol-Content (mol/mol protein)
Cholinesterase	2^2	+	1.6	25
α_2HS-Glycoprotein	–	–	–	–
C_1-Inactivator	2^2	–	0.1	0.5
8Sα_3-Glycoprotein	2^1	–	0.16	1.6
Ig A	2^3	–	0.05	0.4
Inter-α-Trypsin Inhibitor	2^1	–	0.1	0.7
Fetuin*	–	–	0.1	0.2
β_2-Glycoprotein III	–	–	0.1	0.2
Ig D	–	–	–	–

* Fetuin is taken from fetal calf serum

Structure suggested for the receptor of the lectin from *Helix pomatia*: α-N-acetyl-*D*-galactosamine → serine (threonine)

3. Only IgD and α_2HS-glycoprotein have one type of alkali-labile carbohydrate chain, namely the TF-receptor (β-*D*-galactosyl-(1-3)-N-acetyl-*D*-galactosamine) of chain I, whereas all the others also have the chain II type, namely the 'A-like'-N-acetyl-*D*-galactosaminyl-receptor. Both chains can be detected chemically by GLC and serologically by the precipitin reaction or haemagglutination inhibition with specific lectins: all three methods allow a clear cut characterization and estimation of the alkali-labile carbohydrate chains of serum glycoproteins.

Discussion

The classification of serum glycoproteins into two groups, namely those with alkali-labile carbohydrate chains and those lacking them, offers some new aspects for the metabolism, secretion, turnover and the "liver clearance" mechanism as postulated by *Ashwell*'s experiments (fig. 1). It not only contributes new information on the biochemical structure of the carbohydrate part of the different serum glycoproteins, but it raises once again the question of the biological role of the sugar side chains.

One important feature of these asialo carbohydrate chains seems to be that they can be recognized by membrane-integrated vertebrate lectins; these specifically react with the β-galactosyl end-groups, which are normally hidden in a cryptic state (2). Our investigations thus find a solid basis for further studies in this direction. Experiments, which are already in progress, employ radioactive labelled asialo glycoproteins as a means of following their metabolism, their catabolism, their excretion and their clearance by certain organs, for instance the liver.

They also represent a basis for answering the questions, whether the subterminal or next sugars to the β-galactosyl-endgroups have any influence; and whether the anomeric linkage, or the carbohydrate chain itself (alkali-labile or alkali-stable) has anything to do with the vertebrate lectin mediated trap mechanism of these glycosubstances. Finally, they serve as a biological model for studying the metabolism of other (e. g. membrane-bound and isolated membrane glycoproteins) glycosubstances containing one or both types of chains, or one or both of the alkali-labile chains (I and II).

This may also be of great interest with respect to: the trapping of neuraminidase-treated red cells; the "homing" of lymphocytes; the metastases of tumor cells with such a cryptic deletion of carbohydrates or asialo structures; and the interpretation of certain rosette-formation phenomena, in which glycoproteins may participate and membrane lectins may play a role (12).

Knowledge about the structure and distribution of such alkali-labile carbohydrate chains of type I and II, and their different substitution by sialic acids, as reviewed recently (13), must be regarded as the first step towards the biological attitude of investigating their general or special role in different glycosubstances, a spectrum which reaches from the submaxillary mucins to the antifreeze-glycoprotein of the antarctic fishes and to the serum glycoproteins described here (13).

All these relationships, also with respect to the alkali-stable carbohydrate chains, are summarized in figure 2, which includes the biochemical and serological properties of glycoproteins and their "homing" by liver membrane-integrated lectins. Blocking and inhibition studies of the latter will provide further information on the metabolism of glycoproteins; such experiments may also be performed with other glycosubstances of similar structure.

Carbohydrate chains	Alkali-labile	Alkali-labile and alkali-stable	Alkali-stable
Typical examples	Mucin-type, anti-freeze-type	Membrane glycoproteins, Serum glycoproteins	Most serum glycoproteins (see this paper)
End-group after neuraminidase treatment	Gal $\xrightarrow[\beta]{1-3}$ GalNac (I) GalNac $\xrightarrow[\alpha]{}$ Ser / Thr (II)	Gal $\xrightarrow[\beta]{1-4}$ GlcNac	
Trivial name of serological specificity	Anti-TF (I) Anti-A-like (II)	Anti-*pneumococcus* Type XIV cross reactivity	
Lectin receptor (plant and invertebrate)	*Helix pomatia* (II) *Arachis hypogaea* (I) *Agaricus bisporus* (I)	*Phaseolus vulgaris* *Abrus precatorius* *Tridacna maxima*	
Lectin receptor (membrane) (vertebrate)	Liver? inhibition? ←	→ Liver competition?	

Fig. 2. Classification of glycoproteins with respect to their alkali-labile and alkali-stable carbohydrate chains.

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Prof. Dr. G. Uhlenbruck
Abt. Immunbiologie
Kerpener Straße 15
D-5000 Köln 41